Superoxide dismutase and Fenton chemistry

Reaction of ferric–EDTA complex and ferric–bipyridyl complex with hydrogen peroxide without the apparent formation of iron(II)

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A ferric–EDTA complex, prepared directly from $FeCl_3$ or from an oxidized ferrous salt, reacts with H_2O_2 to form hydroxyl radicals ($\cdot OH$), which degrade deoxyribose and benzoate with the release of thiobarbituric acid-reactive material, hydroxylate benzoate to form fluorescent dihydroxy products and react with 5,5-dimethylpyrrolidine N-oxide (DMPO) to form a DMPO–OH adduct. Degradation of deoxyribose and benzoate and the hydroxylation of benzoate are substantially inhibited by superoxide dismutase and $\cdot OH$-radical scavengers such as formate, thiourea and mannitol. Inhibition by the enzyme superoxide dismutase implies that the reduction of the ferric–EDTA complex for participation in the Fenton reaction is superoxide-($O_2\cdot^-$)-dependent, and not H_2O_2-dependent as frequently implied. When ferric–bipyridyl complex at a molar ratio of 1:4 is substituted for ferric–EDTA complex (molar ratio 1:1) and the same experiments are conducted, oxidant damage is low and deoxyribose and benzoate degradation were poorly if at all inhibited by superoxide dismutase and $\cdot OH$-radical scavengers. Benzoate hydroxylation, although weak, was, however, more effectively inhibited by superoxide dismutase and $\cdot OH$-radical scavengers, implicating some role for $\cdot OH$. The iron–bipyridyl complex had available iron-binding capacity and therefore would not allow iron to remain bound to buffer or detector molecules. Most $\cdot OH$ radicals produced by the iron–bipyridyl complex and H_2O_2 are likely to damage the bipyridyl molecules first, with few reacting in free solution with the detector molecules. Deoxyribose and benzoate degradation appeared to be mediated by an oxidant species not typical of $\cdot OH$, and species such as the ferryl ion–bipyridyl complex may have contributed to the damage observed.

INTRODUCTION

Hydroxyl radicals ($\cdot OH$) can be generated in aerobic biological systems by incubating xanthine oxidase with one of its substrates, such as xanthine, hypoxanthine and acetaldehyde, and a suitable iron catalyst. The superoxide ($O_2\cdot^-$) that is formed acts as a reductant for iron and as a precursor of H_2O_2, which is also formed directly in the reaction, and results in an $O_2\cdot^-$-driven Fenton reaction. Any damage done to an added detector molecule is substantially inhibited by the inclusion of either superoxide dismutase or catalase (Fridovich, 1974; McCord & Day, 1978; Halliwell, 1978). When a ferric–EDTA complex is mixed with H_2O_2, Fenton chemistry also takes place (albeit at a much lower rate than with a ferrous–EDTA complex), which damages the detector molecule deoxyribose in a reaction that is substantially inhibited by the addition of superoxide dismutase (Gutteridge, 1985; Gutteridge & Bannister, 1986), suggesting that H_2O_2 does not reduce a ferric–EDTA complex, since such a reductive step would not be inhibited by superoxide dismutase but would be inhibited only by catalase.

Fenton chemistry, in the absence of high-energy radiation, leading to the formation of $\cdot OH$ radicals, is the most likely mechanism for producing a highly oxidizing species in biological systems (for a review see Halliwell & Gutteridge, 1985). Alternatives to the $\cdot OH$ radical have been proposed (Walling, 1982), and there has been renewed interest in the ferryl ion (FeO^{2+}), possessing an oxidation number of four, as a major

contributor to oxidative damage in biological systems (Rush & Koppenol, 1986; Sutton et al., 1987). So far, however, there is no clear evidence for a FeO^{2+} ion outside a haem ring (Kobayashi & Hayashi, 1981; Yusa & Shikama, 1987; Petersen et al., 1989), and haem-associated ferryl has been proposed to account for the ability of myoglobin or haemoglobin mixtures with H_2O_2 to accelerate lipid peroxidation (Kanner et al., 1987).

Attempts to demonstrate damage by the FeO^{2+} ion have been claimed in iron/EDTA systems (Rush & Koppenol, 1986), although we and others have found EDTA to act in a 'radiomimetic' way, releasing substantial numbers of $\cdot OH$ radicals into free solution, where they can be scavenged by $\cdot OH$ scavengers according to their known second-order rate constants (Gutteridge, 1984, 1987; Moorhouse et al., 1985). Indeed, this has been used as a method to measure rate constants (Gutteridge, 1987; Halliwell et al., 1987). When simple iron salts are added to reactions (designed to detect $\cdot OH$ radicals) containing H_2O_2 in the absence of EDTA, most scavengers fail to protect significantly against the damage brought about by Fenton chemistry (Borg & Schaich, 1984; Czapski, 1984). To explain this Gutteridge (1984) has proposed that iron ions weakly bind to the detector molecule to bring about site-specific $\cdot OH$-radical damage and that protection by scavengers is only afforded when the scavengers do protect not by combining with $\cdot OH$ but by binding metal ions. Alternative proposals, however, suggest that damage by simple iron salts is mediated by a crypto $\cdot OH$ radical (Youngman, 1984) or the FeO^{2+} ion (Winterbourn, 1987).

Abbreviation used: DMPO, 5,5-dimethylpyrrolidine N-oxide.
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In the present study we have tested the ability of a ferric–bipyridyl chelate to generate an oxidizing species from H_2O_2. This iron complex is not able to release large numbers of ·OH radicals into free solution, and because of its high affinity for iron would not allow any iron ions to remain bound to the detector molecules with the concentrations studied here. Removal of iron from detector molecules by bipyridyl has been demonstrated experimentally (Aruoma et al., 1989). Under these reaction conditions damage to different detector molecules by ferric–bipyridyl complex dependent on hydrogen abstraction was not effectively inhibited by superoxide dismutase or ·OH-radical scavengers, whereas a weak hydroxylation of benzoate was considerably more responsive to inhibition by ·OH-radical scavengers. These results suggest that, when an iron complex is reacting with H_2O_2 and cannot release ·OH radicals into free solution or site-direct ·OH radicals to a detector molecule, formation and oxidative damage by species other than ·OH may then become more obvious.

MATERIALS AND METHODS

Materials

Catalase (bovine liver; thymol-free), xanthine oxidase (type 1), Nitro Blue Tetrazolium, superoxide dismutase (bovine erythrocyte; specific activity 2800 units/mg of protein), albumin (bovine; fatty acid-free), Ferene S and 2-deoxy-D-ribose were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). H_2O_2 was titrated against thiosulphate using the standard iodometric procedure to determine concentration. 5,5-Dimethylpyrrolidine N-oxide (DMPO) was further purified by vacuum distillation.

Deoxyribose degradation with release of thiobarbituric acid-reactive material

This procedure was carried out essentially as previously described (Gutteridge, 1985). A 0.2 ml portion of 10 mM-deoxyribose, 0.2 ml of phosphate-buffered saline (0.15 M-NaCl/0.10 M-sodium phosphate buffer, pH 7.4), 0.1 ml of iron complex and 0.1 ml of appropriate inhibitor were mixed, and the reaction was started by the addition of 0.1 ml of 5 mM-H_2O_2. Samples were incubated for 1 h at 37 °C.

The ferric–EDTA complex was prepared freshly for each experiment by mixing 2 mM-Fe$(NH_4)_2(SO_4)_2$ with 2 mM-EDTA in the ratio 1.0:1.1 as previously described (Gutteridge, 1985). The EDTA solution (in 0.10 M-sodium phosphate buffer, pH 7.4) and the ferrous salt mixture were left to stand for 1 h to ensure complete conversion of Fe^{2+} ions into Fe^{3+} ions. The absence of Fe^{2+} ions could be verified by adding 2,2-bipyridyl to a sample of the mixture. The iron–bipyridyl complex was prepared freshly for each experiment by mixing 2 mM-bipyridyl and 2 mM-$FeCl_3$ in the ratio 4:1. Both reagents were made up in Chelex-resin-treated distilled water.

Benzoate degradation with the release of thiobarbituric acid-reactive material

On the basis of a previous observation (Gutteridge, 1984), benzoate can be degraded by ·OH radicals to release thiobarbituric acid-reactive material, which can be measured spectrophotometrically or spectrofluorimetrically. The reaction conditions were as described for deoxyribose degradation except that 10 mM-benzoate was substituted for 10 mM-deoxyribose.

Development of thiobarbituric acid-reactivity

After the samples had been incubated at 37 °C for 1 h, 0.2 ml of thiobarbituric acid (1 %, w/v, in 50 mM-NaOH) was added to each tube followed by 0.2 ml of 2.8 % (w/v) trichloroacetic acid. The tubes and their contents were heated for 10 min at 100 °C to develop the pink chromogen. Deoxyribose samples and benzoate reactions in the presence of EDTA were measured spectrophotometrically at 532 nm against appropriate blanks and controls after extraction into 2 ml of butan-1-ol. Benzoate samples in the presence of bipyridyl were extracted with 2 ml of butan-1-ol, and the clear organic upper layer was measured spectrofluorimetrically, after brief centrifugation (1000 g for 5 min), with excitation at 532 nm and emission at 553 nm as previously described (Gutteridge, 1986).

Benzoate hydroxylation to form fluorescent products

The addition of ·OH radicals to benzoate produces fluorescent dihydroxybenzoates, which can be measured spectrofluorimetrically with excitation at 305 nm and emission at 408 nm (Gutteridge, 1987). The reaction conditions were exactly as those described above for benzoate degradation.

Generation of $O_2^{·-}$ radical ions

$O_2^{·-}$ radical ions were generated from xanthine oxidase and hypoxanthine as previously described (Beauchamp & Fridovich, 1970).

E.p.r. spin trapping

DMPO was dissolved in Chelex-resin-treated distilled water and used at a final reaction concentration of 100 mM. The reaction system for spin-trapping experiments was the same as that used for deoxyribose. Samples were incubated for 15 min at 37 °C, then loaded into the tip-sealed capillary of a Pasteur pipette and analysed in an IBM–Bruker ER 300 e.p.r. spectrometer at room temperature (25 °C). Instrumental settings were: modulation frequency, 100 kHz; modulation amplitude, 0.0975 mT; time constant, 81.92 ms; sweep width, 100 G; microwave frequency, 9.75 GHz; power, 19.8 mW.

RESULTS

Previous studies have shown that a ferric–EDTA complex and H_2O_2 generate ·OH radicals, which degrade deoxyribose, by a reaction that is substantially inhibited by superoxide dismutase (Gutteridge, 1985; Gutteridge & Bannister, 1986). Here we confirm and extend those studies to show that this reaction applies to detector molecules other than deoxyribose. The addition of ·OH radicals resulting in the hydroxylation of an aromatic molecule as well as hydrogen abstraction leading to the degradation of deoxyribose and of benzoate, with the release of fragments that react with thiobarbituric acid, occurred in our system (Table 1).

All the ·OH-radical scavengers tested in the three different detector systems, with ferric–EDTA complex and H_2O_2, indicated an important contribution to damage by ·OH radicals. Indeed, a DMPO spin adduct of the ·OH radical was clearly detected in the reaction mixture containing H_2O_2 and ferric–EDTA complex (Fig. 1). Albumin and urea, included as controls for non-specific scavenging effects, were, as expected, without scavenging properties (Table 1).

When a ferric–bipyridyl complex was substituted for the ferric–EDTA complex and exactly the same experiments were conducted, many different results were obtained (Table 2). Deoxyribose and benzoate degradation reactions were poorly, if at all, inhibited by ·OH-radical scavengers, and the effect of superoxide dismutase was not significant. Benzoate hydroxyl-

Table 1. Damage to detector molecules by ferric–EDTA complex and H_2O_2

Final reaction concentrations are shown. The results are means of four or more separate experiments, which differed by less than $\pm 5\%$. Details of reaction mixtures are given in the Materials and methods section. Abbreviation: N.C., no significant change. Heat-denatured-protein controls were included in this study (results not shown).

|	Deoxyribose degradation (thiobarbituric acid-reactivity)		Benzoate degradation (thiobarbituric acid-reactivity)		Benzoate hydroxylation (fluorescence at $\lambda_{em.}$ 408 nm)	
	A_{532}	Inhibition (%)	A_{532}	Inhibition (%)	Fluorescence (arbitrary units)	Inhibition (%)
(1) Blank (no ferric–EDTA) (values subtracted for calculation of percentage inhibition)	0.053		0.025		21	
(2) Control (ferric–EDTA + H_2O_2)	0.752		0.390		1240	
(3) Reaction (2) + superoxide dismutase (0.014 mg/ml)	0.105	86	0.039	90	112	91
(4) Reaction (2) + catalase (0.014 mg/ml)	0.009	99	0.015	96	0	100
(5) Reaction (2) + albumin (0.014 mg/ml)	0.719	N.C.	0.406	N.C.	1290	N.C.
(6) Reaction (2) + formate (14.3 mM)	0.716	77	0.230	41	763	39
(7) Reaction (2) + mannitol (14.3 mM)	0.122	84	0.116	70	393	68
(8) Reaction (2) + thiourea (0.714 mM)	0.049	93	0.050	87	92	93
(9) Reaction (2) + urea (0.714 mM)	0.695	N.C.	0.382	N.C.	1230	N.C.
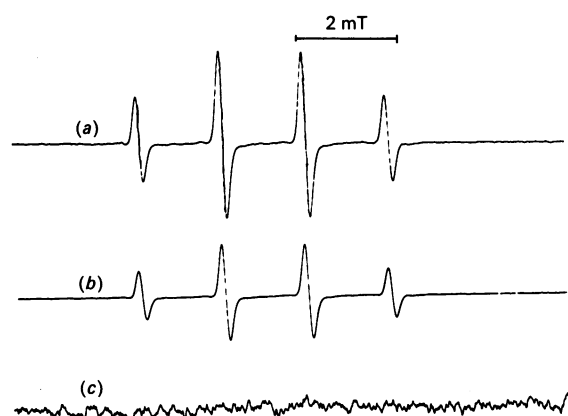
Fig. 1. E.p.r. spectra characteristic of the ·OH-radical adduct observed (a) when $FeCl_3$ and EDTA are mixed with H_2O_2 in the presence of 100 mM-DMPO (gain 2.00×10^5), (b) when $Fe(NH_4)_2(SO_4)_2$ is mixed with EDTA (and allowed to oxidize to ferric–EDTA complex before addition of H_2O_2) and DMPO (gain 1.00×10^5) and (c) with control DMPO in water (gain 2.00×10^6)

ation, a detector system considerably more specific for the formation of ·OH radicals, showed a pattern of inhibition by ·OH-radical scavengers more indicative of a damaging role played by the ·OH radical (Table 2). Thiourea, however, appeared to cause the formation of a fluorescent artifact. During the incubation of the ferric–dipyridyl complex and H_2O_2 continuous spectrophotometric monitoring showed that there was no colour change in the ferric–bipyridyl complex, which indicated that no reduction of Fe^{3+} ions was brought about by H_2O_2. As a control the ferric–bipyridyl complex was incubated with ascorbate instead of H_2O_2 under similar reaction conditions (Fig. 2). The ferrous–bipyridyl complex has a molar absorption coefficient of about 8000 $M^{-1} \cdot cm^{-1}$ at 520 nm (L. Poyer, unpublished work), whereas the compound Ferene S complexed with Fe^{2+} ions has a molar absorption coefficient of 35500 $M^{-1} \cdot cm^{-1}$ at 593 nm (Higgins, 1981). Ferene S was therefore substituted for bipyridyl to monitor at a greater sensitivity the formation of Fe^{2+} ions during the reaction of its ferric complex with H_2O_2 (Fig. 2). No

reduction of either ferric complex was observed in the presence of H_2O_2, but reduction of both ferric complexes did occur in the presence of ascorbate.

The possibility that the ferric–bipyridyl complex might inhibit superoxide dismutase and account for its low protection in two of the reaction systems was investigated by generating $O_2^{·-}$ in the presence of Nitro Blue Tetrazolium and adding ferric–bipyridyl complex to the enzyme. The ferric–bipyridyl complex did not significantly inhibit superoxide dismutase, nor did it inhibit xanthine oxidase used to generate $O_2^{·-}$, as determined by urate formation from hypoxanthine measured at 295 nm.

When compared with ferric–EDTA complex, ferric–bipyridyl complex was a poor Fenton catalyst, giving substantially lower levels of radical damage to the detector molecules (Tables 1 and 2) and a weak e.p.r. signal for the DMPO–OH adduct that was some 20-fold lower than the signal given by ferric–EDTA complex (results not shown). The slow reactivity of bipyridyl with H_2O_2 was shown by mixing 2 mM-bipyridyl and 2 mM-ferrous salt in a ratio of 4:1 and diluting this 1 in 51 with distilled water to give an absorbance of 0.140 at 520 nm. In the presence of 4 mM-deoxyribose, 40 mM-sodium phosphate buffer, pH 7.4, and 0.1 mM-H_2O_2 the absorbance of the bipyridyl–ferrous complex only fell to 0.122 during a 30 min incubation period. This fall in absorbance represents a change in the oxidation state of the iron as the ferrous complex reacts with phosphate and H_2O_2 (Winston et al., 1983).

DISCUSSION

By using the technique of deoxyribose degradation (Gutteridge, 1981; Halliwell & Gutteridge, 1981) it has previously been shown that superoxide dismutase can inhibit the EDTA-dependent $O_2^{·-}$-driven Fenton reaction at two different stages (Gutteridge, 1985; Gutteridge & Bannister, 1986). The enzyme inhibits the first stage by preventing $O_2^{·-}$ from reducing an Fe^{3+}–EDTA complex and in the second stage prevents formation of ·OH radicals during the reaction of ferric–EDTA complex with H_2O_2, the product of the dismutation reaction; see simplified reactions (1), (2) and (3):
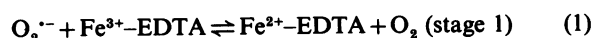
$$O_2^{·-} + Fe^{3+}\text{–EDTA} \rightleftharpoons Fe^{2+}\text{–EDTA} + O_2 \text{ (stage 1)} \qquad (1)$$

Table 2. Damage to detector molecules by ferric–bipyridyl complex and H_2O_2

Final reaction concentrations are shown. The results are means of four or more separate experiments, which differed by less than ±5%. Details of reaction mixtures are given in the Materials and methods section. Abbreviation: N.C., no significant change. Heat-denatured-protein controls were included in this study (results not shown).
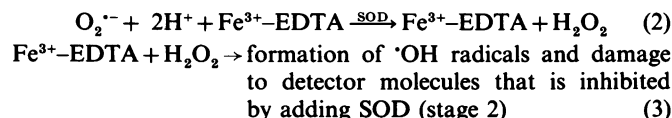
	Deoxyribose degradation (thiobarbituric acid-reactivity)		Benzoate degradation (thiobarbituric acid-reactivity)		Benzoate hydroxylation (fluorescence at $\lambda_{em.}$ 408 nm)	
	A_{532}	Inhibition (%)	A_{532}	Inhibition (%)	Fluorescence (arbitrary units)	Inhibition (%)
(1) Blank (no ferric–bipyridyl) (values subtracted for calculation of percentage inhibition)	0.043		20		4.7	
(2) Control (ferric–bipyridyl + H_2O_2)	0.116		59		8.0	
(3) Reaction (2) + superoxide dismutase (0.014 mg/ml)	0.124	N.C.	57	N.C.	3.4	58
(4) Reaction (2) + catalase (0.014 mg/ml)	0.008	93	0	100	0.3	96
(5) Reaction (2) + albumin (0.014 mg/ml)	0.126	N.C.	55	N.C.	8.7	N.C.
(6) Reaction (2) + formate (14.3 mM)	0.124	N.C.	54	N.C.	4.0	50
(7) Reaction (2) + mannitol (14.3 mM)	0.121	N.C.	59	0	4.3	44
(8) Reaction (2) + thiourea (0.714 mM)	0.095	18	61	N.C.	*	*
(9) Reaction (2) + urea (0.714 mM)	0.126	N.C.	57	N.C.	8.0	0

* A fluorescence value of 22 units representing 180% stimulation was obtained for thiourea. This appears to be a reproducible artefact within this reaction.
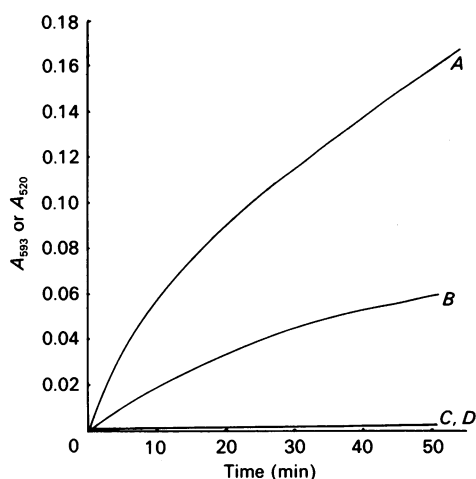
Fig. 2. Reduction of iron complexes

Curve A, reduction of ferric–Ferene S complex (measured at 593 nm) by ascorbate (0.0625 mM); curve B, reduction of ferric–bipyridyl complex (measured at 520 nm) by ascorbate (0.0625 mM); curve C, D, reduction of ferric–Ferene S complex and of ferric–bipyridyl complex by H_2O_2 (0.7 mM). The reaction conditions were as described for deoxyribose degradation.

Reaction (1) plus superoxide dismutase (SOD) proceeds to reaction (2):

$$O_2^{\cdot-} + 2H^+ + Fe^{3+}\text{-EDTA} \xrightarrow{SOD} Fe^{3+}\text{-EDTA} + H_2O_2 \quad (2)$$

Fe^{3+}-EDTA + $H_2O_2 \rightarrow$ formation of ·OH radicals and damage to detector molecules that is inhibited by adding SOD (stage 2) (3)

The implications of superoxide dismutase inhibiting this second stage is that the eventual formation of ·OH radicals from ferric–EDTA complex and H_2O_2 appears to be almost completely
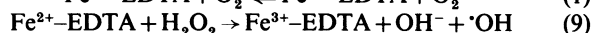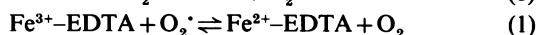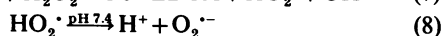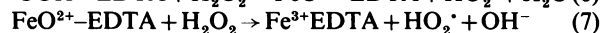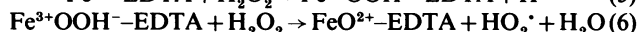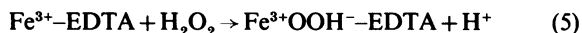
$O_2^{\cdot-}$-dependent. This observation is confirmed and extended here to include detector molecules other than deoxyribose and radical damage involving both hydrogen abstraction as well as aromatic hydroxylation. The metal-ion chelator EDTA provides a useful tool for studying Fenton chemistry in aqueous solution, since it has several important properties, which include a radiomimetic effect (Gutteridge, 1987), the ability to keep iron soluble and reactive in solution (Flitter et al., 1983), advantageously altering the redox potential of iron, allowing the soluble complex to participate in both the oxidative and reductive steps of Fenton chemistry (Grootveld & Halliwell, 1986), and inhibition of the participation of copper ions in Fenton reactions. Disadvantages, however, of using EDTA are that it may be idiosyncratic and not represent a model for a biological iron complex and that it does not readily indicate the oxidation state of the iron complexed to it. Almost complete inhibition of the formation of ·OH radicals in the ferric–EDTA complex–H_2O_2 reaction by superoxide dismutase suggests that there is no direct reduction of ferric–EDTA complex by H_2O_2, since the participation of Fe^{2+} ions formed this way in Fenton chemistry would not be inhibitable by superoxide dismutase. The iron chelators 2,2-bipyridyl and Ferene S, which have characteristic absorption spectra when complexed to Fe^{2+} ions, did not show a colour change during their incubation with H_2O_2, suggesting that they too may not be reduced by H_2O_2. The reaction of a ferric salt with H_2O_2 is frequently represented in the literature as eqn. (4) (Uri, 1952; Walling et al., 1975):

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\cdot} + H^+ \quad (4)$$

This may summarize a complex reaction sequence but is incorrect for EDTA-chelated Fe^{3+} ions if it is used to imply a direct reduction of Fe^{3+} ion by H_2O_2. It has already been suggested that H_2O_2 is unlikely to reduce complexed iron (Melnyk et al., 1981; Wood, 1988), and if it did occur in the reaction with ferric–EDTA complex then superoxide dismutase would only partly inhibit reactions starting with the EDTA equivalent of sequence (4) leading to the formation of ·OH radicals. On the basis of proposals for the reaction of haem iron and H_2O_2 put forward by Petersen et al. (1989), we tentatively suggest that a similar

sequence might explain our $O_2^{\cdot-}$-dependent Fenton reaction for EDTA-chelated iron. These reactions are summarized in eqns. (5)–(9):

$$Fe^{3+}\text{-}EDTA + H_2O_2 \rightarrow Fe^{3+}OOH^-\text{-}EDTA + H^+ \quad (5)$$
$$Fe^{3+}OOH^-\text{-}EDTA + H_2O_2 \rightarrow FeO^{2+}\text{-}EDTA + HO_2^{\cdot} + H_2O \quad (6)$$
$$FeO^{2+}\text{-}EDTA + H_2O_2 \rightarrow Fe^{3+}EDTA + HO_2^{\cdot} + OH^- \quad (7)$$
$$HO_2^{\cdot} \xrightarrow{pH\,7.4} H^+ + O_2^{\cdot-} \quad (8)$$
$$Fe^{3+}\text{-}EDTA + O_2^{\cdot} \rightleftharpoons Fe^{2+}\text{-}EDTA + O_2 \quad (1)$$
$$Fe^{2+}\text{-}EDTA + H_2O_2 \rightarrow Fe^{3+}\text{-}EDTA + OH^- + \cdot OH \quad (9)$$

The ferric–EDTA complex has an 'open structure' (Halliwell & Gutteridge, 1986) and allows a substantial number of $\cdot OH$ radicals to escape into free solution, thus mimicking high-energy radiolysis of water (radiomimetic effect) (Gutteridge, 1987). The ferryl ion–EDTA complex, if formed as depicted in eqn. (6), appears to make little contribution to the damage detected, since it would still be available in the reaction in the presence of superoxide dismutase. An alternative explanation is that it is indistinguishable in all aspects from the $\cdot OH$ radical, a conclusion that so far has not been experimentally established.

A ferric–EDTA complex prepared by the oxidation of ferrous–EDTA complex was found to be more effective in some radical reactions leading to deoxyribose degradation than a complex prepared directly from a ferric salt (Gutteridge, 1985); it also gave a slightly greater DMPO–OH signal. The oxidized complex was used in these studies because of the low sensitivity of the benzoate-degradation method. Although the iron–EDTA complex prepared by oxidation of ferrous–EDTA complex is known to be in the ferric state, it has e.p.r. and Mössbauer spectroscopic properties distinct from those of an iron–EDTA complex prepared from a ferric salt (Marton et al., 1987). However, both ferric–EDTA preparations are substantially inhibited by superoxide dismutase (Gutteridge, 1985; Gutteridge & Bannister, 1986) and generate a DMPO–OH adduct in the presence of H_2O_2 (Fig. 1).

Bipyridyl was mixed with a ferric salt at a ratio of 4:1 so that a considerable iron-binding capacity was still present and no iron could remain on any of the detector molecules used and bring about the site-specific formation of $\cdot OH$ radicals. Such binding has previously been shown to give rise to damage that cannot be inhibited by $\cdot OH$-radical scavengers unless they themselves have greater iron-binding properties than the detector molecule (Gutteridge, 1984). The 'closed' structure of the iron–bipyridyl complex does not appear to release large numbers of $\cdot OH$ radicals into free solution. Most $\cdot OH$ radicals generated by the complex would be expected to react with the bipyridyl molecules in a way analogous to the reaction of pyrroles with $\cdot OH$ radicals in haem complexes, making it difficult to obtain clear patterns of inhibitions, with scavenger studies, similar to those seen for EDTA. The poor release of $\cdot OH$ into free solution by the ferric–bipyridyl complex, compared with the ferric–EDTA complex, can be seen from the extents of aromatic hydroxylation of benzoate. This may in part reflect the higher ratio of chelator to iron salt present and the slow reaction of ferric–bipyridyl complex with H_2O_2. Fig. 3 shows the possible 'closed' structure of the ferric–bipyridyl complex, which would be expected to scavenge most $\cdot OH$ radicals generated at the iron centre. When the ratio of iron salt to bipyridyl is lowered to 1:3 and the concentration of $\cdot OH$-radical scavengers is increased from 14 mM to 50 mM, some inhibition of damage to deoxyribose is then observed (Gutteridge, 1990), suggesting that $\cdot OH$ radicals can be intercepted in the reaction system.

Damage to deoxyribose and benzoate by hydrogen abstraction, resulting in the release of thiobarbituric acid-reactive material, when ferric–bipyridyl complex and H_2O_2 were added was not characteristic of the $\cdot OH$ radical in free solution or at a specific
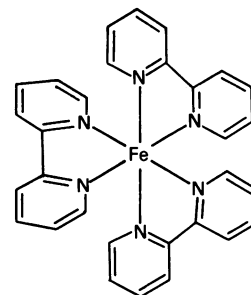

Fig. 3. Representation of the 'closed' structure of ferric–bipyridyl complex

site on the detector molecule dictated by iron binding. When the more specific technique of $\cdot OH$ detection based on the aromatic hydroxylation of benzoate was measured, however, there was a weak but detectable increase in fluorescence that could partly be inhibited by $\cdot OH$-radical scavengers, suggesting some involvement of the $\cdot OH$ radical in this damage.

Ferric–bipyridyl complex reacts with H_2O_2 and appears to produce an oxidizing species, in addition to the $\cdot OH$ radical, which could be the ferryl ion–bipyridyl complex. This may be able to damage detector molecules by a reaction similar to hydrogen abstraction, but is probably unable to hydroxylate aromatic compounds, hence the different patterns of inhibition obtained with the three detector molecules. A similar observation has been reported for a mixture of ferrous haemoglobin and H_2O_2 (Puppo & Halliwell, 1988), which is able to degrade deoxyribose but unable to hydroxylate an aromatic detector molecule. The iron–bipyridyl complex may provide a useful model for studying reactions of haem iron complexes with H_2O_2.

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